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13. ABSTRACT ***(Maximum 200 Words)***

DNA is tightly packed into the eukaryotic nucleus in the form of chromatin. Chromatin is built of nucleosomal repeat units comprised of histone proteins and DNA. Nucleosomes are folded together into higher order structures that inhibit transcription. This proposal tests the idea that an enzyme that regulates chromatin folding through post-translational modification of the histones is important for p53 functions and for estrogen responses, as well as for normal breast development. Experiments are proposed to 1) determine whether Gcn5 serves as a coactivator for activation of gene expression by the estrogen receptor 2) examine biochemical, molecular, and genetic connections between Gcn5 and p53 and 3) to generate a mammary gland specific 'knock out' of Gcn5 in mice to create a mouse model for Gcn5 functions in breast development and tumor formation. We have made progress towards all three aims. We have created cell lines carrying stable reporter genes carrying estrogen response elements assembled into chromatin. We have uncovered biochemical and genetic connections between Gcn5 and p53. We have generated a conditional disruption allele for Gcn5. Together these studies should provide important new information about breast cancer biology. Moreover, histone acetyltransferases may also provide novel targets in the future for development of new drug therapies or diagnostic agents, furthering our advancement towards eradication of this disease.

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Table of Contents

INTRODUCTION:

The compaction of DNA into chromatin in the eukaryotic nucleus limits transcription by limiting access of the basal transcription machinery and regulatory proteins to their target sequences (Edmondson and Roth, 1996). Chromatin remodeling is now recognized as a central feature of gene regulation. Two major classes of chromatin remodeling activities have been identified to date (Struhl, 1998; Wade and Wolffe, 1999). One class includes large ATP-driven complexes typified by Swi/Snf, which regulate nucleosome placement and movement. The second class is comprised of enzymes responsible for regulating post-translational modifications of the histone proteins. Of these, histone acetyltransferases (HATs) and histone deacetylases (HDACs) are best characterized (Roth et al., 2001). Particular HATs, such as AIB1, have been implicated in cancers, including breast cancer (Anzick et al., 1997). Other HATs are required as cofactors for tumor suppressor activities such as p53 or for hormonal responses (Blanco et al., 1998; Liu et al., 1999; Schiltz and Nakatani, 2000). The GCN5 HAT is highly related to PCAF (Xu et al., 1998), which has been linked both to p53 and estrogen receptor functions. This proposal seeks to understand the role of GCN5 in these processes and to determine whether GCN5 serves as a suppressor or enhancer of breast tumor formation. The importance of GCN5 to normal breast development will be determined as well. The proposal has three specific aims: 1) to determine whether GCN5 serves as a coactivator for the estrogen receptor 2) to examine biochemical, molecular and genetic connections between GCN5 and p53 and 3) to generate a mammary gland specific 'knock out' of GCN5 in mice. The results of these studies may provide new targets for therapies or novel diagnostic tools.

BODY:

Our Statement of Work is organized into three tasks. We have made good progress on each of these tasks in this first year of funding, as described below:

Task1: To determine whether GCN5 serves as a coactivator for the estrogen receptor in transcriptional activation in mammalian cells.

- Goal 1: Transfect NIH 3T3 cells with expression constructs for the estrogen receptor (ER), FLAG-tagged GCN5, and an estrogen responsive reporter gene. Monitor expression of the reporter gene in the presence and absence of estradiol.

  Progress: Completed. Unfortunately, the results of these transient transfection experiments were inconclusive and suggested to us that we needed to create stable cell lines carrying estrogen responsive reporter genes integrated into the chromosome and that we needed to examine additional types of cells. To this end, we have selected stable cell lines (MCF7 and CV1 cells) that carry an integrated reporter gene and are now in the process of assaying the ability of GCN5 to participate in the activation of this gene by the ER.

- Goal 2: Construct mutated forms of GCN5 for transfection experiments to determine the domains required for estrogen mediated functions.

  Progress: We have constructed point mutations in the GCN5 HAT catalytic center, and will test these in the above cell lines soon. Further definition of domains involved in estrogen responses will be determined in the coming year, if we see a synergism between GCN5 and the ER.

- Goal 3: Perform in vitro binding experiments to determine if GCN5 interacts directly with the estrogen receptor.

Progress: These experiments are on hold until (or unless) we demonstrate some synergistic activation effects in the above experiments.

- Goal 4: Perform co-immunoprecipitation experiments to determine if GCN5 is associated (directly or indirectly) with the estrogen receptor in vivo.

  Progress: On hold pending the outcome of the above experiments.

Task 2: To examine the role of GCN5 in p53 functions.

- Goal 1: Express and purify recombinant p53.

  Progress: Completed.

- Goal 2: Test recombinant p53 as a substrate for recombinant GCN5.

  Progress: Completed. We found that GCN5 can acetylate p53 in vitro (Fig. 1) and are currently determining which domains in p53 are acetylated.

- Goal 3: Perform co-transfection experiments to determine if GCN5 augments p53 transactivation in vivo, and if so, determine which domains in GCN5 are required for this effect.

  Progress: Completed. Unfortunately, we could never demonstrate any augmentation of p53 responses upon cotransfection with GCN5 in transient transfection experiments (see Fig. 2 for example). This lack of response may indicate that GCN5 functions may only be required when the reporter gene is packaged into chromatin. Therefore we examined expression of the endogenous p21 gene, which is p53 responsive. Again we saw no effect of addition of GCN5 (data not shown). Since we do not have any cell lines that lack enodgenous GCN5, it may be that endogenous levels of this enzyme are obscuring any effects of the transfected GCN5. Therefore we are hoping to create a 'dominant negative' form of GCN5 in the coming year.

- Goal 4: Cross mice heterozygous for the GCN5 null allele with mice homozygous for the p53 allele.

  Progress: Completed. We have successfully generated mice that are null for p53 and heterozygous for the GCN5 null allele. We are continuing our breeding to generate more of these mice, as well as mice that heterozygous for both the p53 null allele and the Gcn5 null allele.

- Goal 5: Cross offspring from the above matings to generate mice that are null for both p53 and GCN5, to see if loss of p53 rescues the embryonic lethality resulting from GCN5 loss.

  Progress: In progress and on track for completion in year 2. After several months of breeding, we have never recovered any live pups that are null for p53 and for GCN5. All other expected genotypes were recovered, but mice null for p53 and heterozygous for GCN5 were under represented. We are now examining embryos from these crosses to determine if p53 loss allows the GCN5 null mice to develop further than usual.

Task 3: To generate a mammary gland-specific 'knock out' of GCN5 in mice.

- Goal 1: Construct targeting vector for GCN5 gene replacement.

Progress: Completed (Fig. 3).

- Goal 2: Transform targeting vector in ES cells and select/screen properly targeted cells.

  Progress: Completed. We identified 7 independent clones that carry the properly targeted replacement allele (Fig. 3, for example).

- Goal 3: Inject above ES cells into blastocysts to generate chimeric mice.

  Progress: in progress. Blastocysts are being injected this month, and we anticipate birth of our first chimeras in the next 6-8 weeks.

- Goal 4: Breed chimeric mice with wild type mice to generate mice heterozygous for the replacement allele.

  Progress: We will begin these crosses as soon as our chimeras are old enough to breed.

- Goal 5: Breed heterozygous mice with each other to generate mice homozygous for the replacement allele.

  Progress: On track. We should start these crosses in the next 6 months, as soon as we obtain the heterozygotes in the above goal.

- Goal 6: Breed homozygous mice with WAP-cre transgenic mice.

  Progress: On track for completion during year 2.

- Goal 7: Analyze GCN5 removal and mammary gland development and mammary tumor formation in female mice resulting from the above croos before, during, and after pregnancy.

  Progress: On track for completion during year 3 of the grant.

KEY RESEARCH ACCOMPLISHMENTS:

- Creation of stable cell lines carrying integrated estrogen responsive reporter genes.
- Demonstration that GCN5 can acetylate p53 in vitro.
- Determination that loss of p53 does not rescue the lethality of loss of GCN5.
- Construction of a targeting vector for creation of a breast-specific knock out of GCN5 in mice.
- Proper targeting of this replacement allele in ES cells.

REPORTABLE OUTCOMES:

This first year we have made good progress towards our goals, but our work is not yet at a suitable stage for publication. We anticipate publications in the next two years that will report the cell lines and the mice that we create, as described above.

CONCLUSIONS:

We have determined that transient transfection experiments are limited in their ability to delineate the role of GCN5 as a coactivator in p53 or ER responses. We have adapted our strategy accordingly to examine either endogenous genes regulated by these factors, or stable, integrated reporter genes. We are also expanding our experiments to include creation of dominant negative forms of GCN5, which may be able to inhibit ER or p53 responses. These studies are important to understanding both tumor suppression by p53 and tumor enhancement by estrogen. They may reveal new ways of diagnosing breast tumors or new strategies for treatment. In addition, our last task will provide novel insights into the role of chromatin modifying activities in normal breast development as well as in tumor formation.

REFERENCES:

Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer, Science *277*, 965-8.

Blanco, J. C. G., Minucci, S., Lu, J., Yang, X.-J., Walker, K. K., Chen, H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998). The histone acetylase PCAF is a nuclear receptor coactivator, Genes and Devel *12*, 1638-1651.

Edmondson, D. G., and Roth, S. Y. (1996). Chromatin and transcription, FASEB Journal *10*, 1173-82.

Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., and Berger, S. L. (1999). p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage, Mol Cell Biol *19*, 1202-1209.

Roth, S. Y., Denu, J. M., and Allis, C. D. (2001). Histone Acetyltransferases, Annu Rev Biochem *70*, 81-120.

Schiltz, R. L., and Nakatani, Y. (2000). The PCAF acetylase complex as a potential tumor suppressor, Biochim Biophys Acta *1470*, M37-53.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms, Genes and Dev *12*, 599-606.

Wade, P. A., and Wolffe, A. P. (1999). Transcriptional regulation: SWItching circuitry, Curr Biol *9*, R221-4.

Xu, W., Edmondson, D. G., and Roth, S. Y. (1998). Mammalian GCN5 and P/CAF acetyltransferases share homologous amino-terminal domains important for the recognition of nucleosomal substrates, Mol Cell Biol *18*, 5659-5669.

APPENDIX

Figure Legends (one page)

Three Figures (1, 2, 3)

Figure Legends.

Fig. 1. GCN5 acetylates p53 in vitro.

Recombinant murine GCN5 purified from bacteria was incubated with histones (positive control), BSA (negative control), recombinant p53, or recombinant D30p53 (as indicated) under standard HAT reaction conditions using 0.2 μCi ^{3}H-acetyl CoA (see Brownell and Allis, PNAS 92: 6364-6368, 1995). Acetylated (tritiated) products are separated by SDS PAGE and are visualized by fluorogram as shown here. As expected, GCN5 acetylated histones H3 and H4. GCN5 also acetylated full length p53, but not the D30p53 derivative which is missing previously identified PCAF acetylation sites. The last two lanes are controls, showing no tritium incorporation (i.e., no acetylation) in the absence of the GCN5 enzyme.

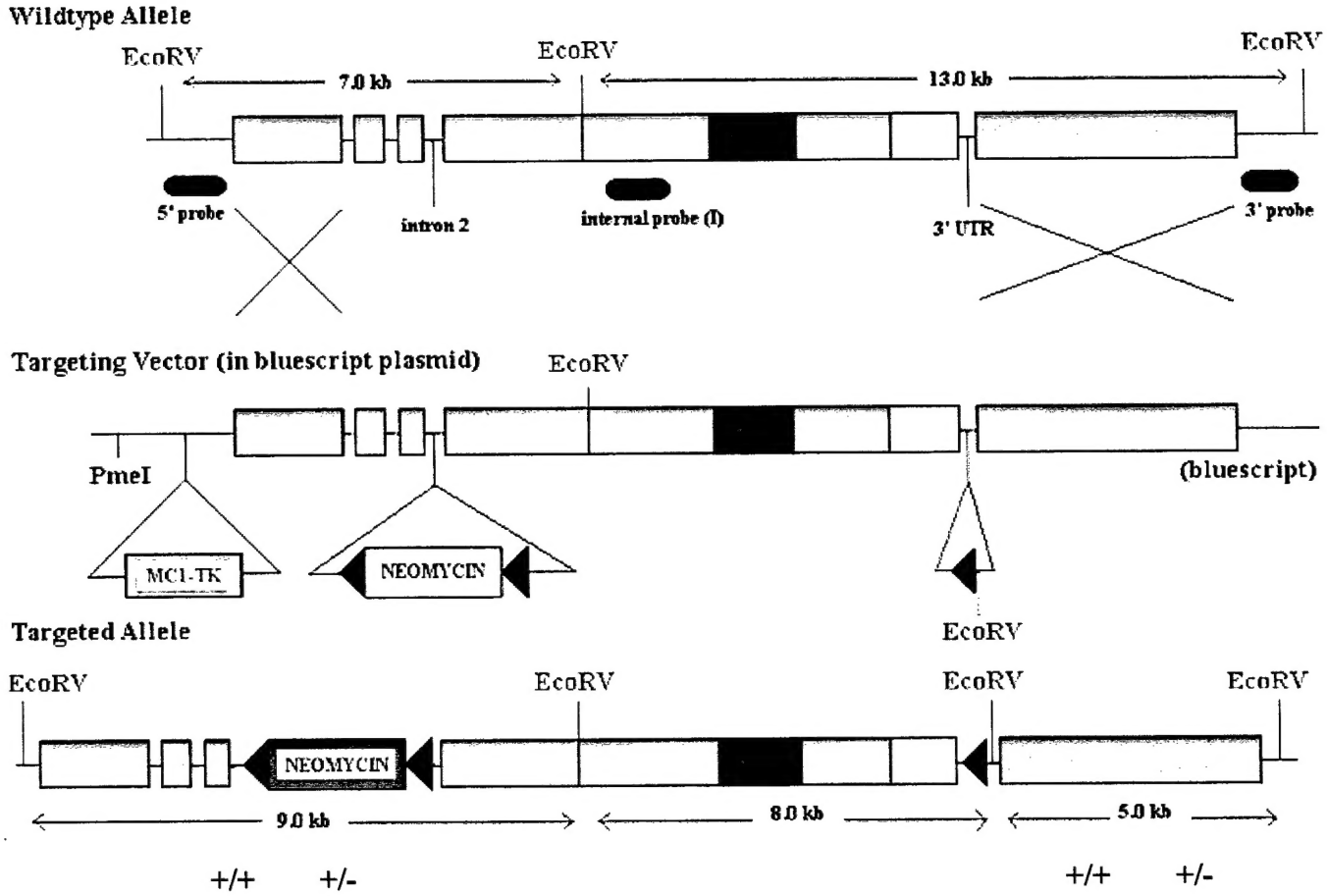
Fig. 2 GCN5 does not enhance p53 activation.

Cultured H1299 cells, which do not express p53, were transiently transfected with a p21-luciferase reporter gene and two different amounts of a p53 (5 and 50 ng) expression vector alone or in combination with two different amounts of a GCN5 expression vector (100 and 500 ng). Expression of both p53 and GCN5 in the transfected cells was confirmed by western blot (data not shown). Reporter gene activation was measured by assay of luciferase activity. Shown are the averages of triplicate samples for each transfection. Although increasing luciferase expression was detected with increasing amounts of p53 (compare 1st, 2nd, and 5th bars), no additional activation was observed upon addition of GCN5.

Fig. 3. Targeting of the GCN5 locus in embryonic stem cells.

The targeting strategy and targeting vectors are shown in the diagrams, and Southern blots comfirming proper targeting of the GCN5 locus in ES cells are shown below. The blue areas in the diagram represent regions of GCN5 flanking sequence cloned into the targeting vector. These arms of homology serve as the donors for the targeted recombination events, as indicated by the crosses (X). The placement of the MC1-TK gene, the neomycin cassette, and the loxP recombination sites (red triangles) are indicated. The GCN5 coding region is indicated in green, with the HAT domain highlighted in red. Upon digestion with EcoRV, the wild type GCN5 locus yields a 7 kb fragment detected by the 5' probe, whereas the targeted allele yields a 9 kb fragment. The 3' probe detects a 13 kb band from the wild type allele, and a 5 kb band from the targeted allele. Properly targeted ES cells should be heterozygous (+/-), yielding one wild type

and one targeted GCN5 band, as confirmed by the Southern blots. As expected, non-targeted cells (+/+) yield only the wild type bands.



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